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Hanson, James V M ; Hediger, Michael ; Manogaran, Praveena ; Landau, Klara ; Hagenbuch, Niels ; Schippling, Sven ; Gerth-Kahlert, Christina

Abstract: Purpose Recent evidence suggests structural changes distal to the inner retina in multiple sclerosis (MS) patients. The functional correlates of these proposed structural abnormalities remain unclear. We investigated outer retinal function and structure in MS patients, and quantified to what extent outer retinal structure influenced function in these patients. Methods Outer retinal function was assessed using the full-field and multifocal electroretinogram (ERG/MF-ERG), whereas retinal structure was assessed using spectral-domain optical coherence tomography (OCT). Results were compared with preexisting normative data. The relationships between electrophysiology parameters and the OCT values corresponding to the proposed cellular origins of the ERG and MF-ERG were analyzed. Results Most electrophysiological responses were delayed in MS patients, independently of optic neuritis (ON). Inner retinal thickness and volumes were reduced, and inner nuclear layer volume marginally increased, in eyes with previous ON; all other OCT parameters were normal. OCT results correlated with ERG amplitudes, but not with ERG peak times or any MF-ERG parameters. Conclusions We recorded outer retinal dysfunction without detectable abnormalities of the corresponding retinal layers in MS patients, not ascribable to retrograde degeneration following ON. The findings complement a growing body of literature reporting primary retinal abnormalities distal to the ganglion cell-inner plexiform layer complex in MS patients, with our data suggesting that this may be a more widespread phenomenon than previously thought. ERG may be of more utility in detecting retinal dysfunction in MS patients than MF-ERG. Analysis of peak times, rather than response amplitudes, is recommended.

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Outer Retinal Dysfunction in the Absence of Structural Abnormalities in Multiple Sclerosis

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Purpose. Recent evidence suggests structural changes distal to the inner retina in multiple sclerosis (MS) patients. The functional correlates of these proposed structural abnormalities remain unclear. We investigated outer retinal function and structure in MS patients, and quantified to what extent outer retinal structure influenced function in these patients.

Methods. Outer retinal function was assessed using the full-field and multifocal electroretinogram (ERG/MF-ERG), whereas retinal structure was assessed using spectral-domain optical coherence tomography (OCT). Results were compared with preexisting normative data. The relationships between electrophysiology parameters and the OCT values corresponding to the proposed cellular origins of the ERG and MF-ERG were analyzed.

Results. Most electrophysiological responses were delayed in MS patients, independently of optic neuritis (ON). Inner retinal thickness and volumes were reduced, and inner nuclear layer volume marginally increased, in eyes with previous ON; all other OCT parameters were normal. OCT results correlated with ERG amplitudes, but not with ERG peak times or any MF-ERG parameters.

Conclusions. We recorded outer retinal dysfunction without detectable abnormalities of the corresponding retinal layers in MS patients, not ascribable to retrograde degeneration following ON. The findings complement a growing body of literature reporting primary retinal abnormalities distal to the ganglion cell–inner plexiform layer complex in MS patients, with our data suggesting that this may be a more widespread phenomenon than previously thought. ERG may be of more utility in detecting retinal dysfunction in MS patients than MF-ERG. Analysis of peak times, rather than response amplitudes, is recommended.

Keywords: retina, multiple sclerosis, optic neuritis, electrophysiology, optical coherence tomography

Multiple sclerosis (MS) is a complex, heterogeneous neurologic disorder characterized by inflammatory demyelination and degeneration within the central nervous system. The near-ubiquity of visual system involvement in MS[1,2] and unique accessibility of the retina as a site for viewing unmyelinated axons and neurons in vivo have driven interest in the afferent visual pathway as a clinical model for MS research.[3] Accordingly, spectral-domain optical coherence tomography (OCT) has become a widely used tool to assess neuronal and axonal degeneration in MS patients.[4] However, OCT remains a structural measure; it is currently not possible to measure retinal neuronal function using OCT in a manner analogous to functional magnetic resonance imaging (MRI) of the brain. Instead, retinal function can be measured using electrophysiology, particularly the ERG and its variants. For example, it is possible to measure the function of the photoreceptors and bipolar cells from the entire or localized areas of central retina using the ERG,[5,6] and multifocal ERG (MF-ERG),[7] respectively.

In recent years, the question of retinal pathology distal to the inner retina in MS has gained increasing interest. For example, postmortem histologic analysis has revealed evidence of atrophy of the inner nuclear layer (INL) in addition to the inner retinal layers (both the retinal ganglion cells and their axons, found in the retinal nerve fiber layer [RNFL]).[8] Additionally, INL has recently been described as responding dynamically to disease activity and treatment; untreated MS patients were found to have a greater INL volume than healthy control subjects, yet this volume appeared to normalize following successful disease-modifying therapy (as evidenced by no clinical relapses or new MRI lesions during the follow-up period).[9] It has also been proposed that an atypical subset of MS patients may exhibit thinning of the INL and outer nuclear layer (ONL) without corresponding inner retinal changes.[10] The functional correlates of these proposed structural changes remain unknown; although electroretinographic studies of MS patients have been previously published,[11–13] results vary considerably between studies, and to date no published work has examined the relationships between OCT-derived outer retinal structure and function in MS.

Against this background, we have embarked on a longitudinal study of retinal structure and function in MS patients.

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Here, we present the results of a cross-sectional analysis of baseline measurements. We aimed to ascertain whether outer retinal function and structure are abnormal in a representative MS and clinically isolated syndrome (CIS) cohort, and to investigate whether retinal function is related to OCT-derived structural measures in MS patients. Finally, we aimed to generate hypotheses as to which (if any) electrophysiological parameters are most suitable for further analysis.

Materials and Methods

All MS patients were participants in the prospective longitudinal Heterogeneity of Multiple Sclerosis (HETOMS) study at the University Hospital Zurich and University of Zurich who consented in writing to additionally participate in a longitudinal ophthalmologic substudy. Inclusion criteria for the substudy were as follows: confirmed diagnosis of MS or CIS,[14] and age at enrollment 18 to 65 years. Exclusion criteria were as follows: refractive errors >6 diopters, coexisting ocular or neurologic disease other than MS, and diabetes mellitus. The study adhered to the tenets of the Declaration of Helsinki and was approved by the Cantonal Ethics Committee of Zurich (EC-No.2013-0001). All patients received a comprehensive eye examination including best-corrected high- and low-contrast visual acuity using Early Treatment Diabetic Retinopathy Study (ETDRS) and 2.5% contrast Sloan charts, respectively, anterior segment and mydriatic fundus examination by a senior ophthalmologist (CG-K), spectral-domain OCT (Spectralis, Heidelberg Engineering, Heidelberg, Germany), ERG, and MF-ERG. Although the study was primarily focused on the outer retina, pattern-reversal visual-evoked potentials, pattern ERG, and the photopic negative response were also measured (as described in Supplementary Data) to assess and compare visual pathway and retinal ganglion cell function.

Optical Coherence Tomography

High-resolution circumpapillary scans (12° diameter; 100 Automatic Real-time Tracking [ART] scans averaged) were aligned to the visible center of the optic nerve head, whereas high-resolution volume scans (30° vertical by 15° horizontal pattern composed of 19 vertically oriented sections, each separated by 240 μm, 25 ART) were centered on the fovea. After ensuring that all acquired OCT scans were of acceptable quality as defined by the OSCAR-IB criteria,[15] the volume scans were automatically segmented and manually verified using proprietary software (Heidelberg Engineering). This enabled visualization and quantification of the macular ganglion cell–inner plexiform layer complex (GC-IPL), INL, outer plexiform layer (OPL), ONL, and photoreceptor (PR) complex for each eye (Fig. 1a). Each of the macular OCT parameters was summarized as the volume (in mm^3) of each layer or complex measured over the 1, 2.22, and 3.45 mm ETDRS grid. RNFL thickness measurements were obtained from the circumpapillary OCT scan (Fig. 1b); the global thickness (RNFL-G), averaged from all sectoral measurements, was analyzed, along with thickness in the temporal (RNFL-T) and papillomacular bundle (RNFL-PMB) sectors as implemented in the software (Fig. 1c).

Electrophysiology

ERG and MF-ERG were recorded using gold-plated skin electrodes and single-use DTL (Dawson, Trick, and Litzkow)-type recording electrodes (Diagnosys LLC, Lowell MA, USA) according to published standards of the International Society for Clinical Electrophysiology of Vision[5,16] on an Espion system (Diagnosys LLC). Medical mydriasis was accomplished using topical 0.5% tropicamide and 5% phenylephrine. Before applying the skin electrodes (reference electrodes at the ipsilateral outer canthi; ground electrode at the center of the forehead), patients' skin was first cleaned and then scrubbed using OneStep AbrasivPlus paste (H + H Medizinprodukte GbR, Münster, Germany) to minimize electrical impedance during recording. To prevent potential patient discomfort, 0.4% oxybuprocaine was instilled before positioning the DTL electrodes.

Before recording the ERG, patients were dark-adapted for 20 minutes. When this adaptation period was over, patients were presented with 0.01 cd/m^2 flashes ("rod") followed by 3.0 cd/m^2 flashes ("rod-cone") to measure the responses of the rod system and combined rod-cone systems, respectively. When these measurements were complete, patients were adapted to a rod-bleaching 30 cd/m^2 light for 10 minutes before being presented with 3.0 $cdm/^2$ light, both flickering (30-Hz frequency; "cone flicker") and single flashes ("cone"). All stimuli were presented via a full-field stimulator with diffusor, of 4-ms duration, and composed of white light. Multiple responses were recorded to verify reproducibility, which were averaged to produce the final responses from which parameters for analysis were derived (rod and rod-cone ERG: average of 6 responses; flicker ERG: average of 26 responses; cone ERG: average of 24 responses).

The MF-ERG was recorded in normal room illumination following the ERG using an achromatic 61-hexagon stimulus array covering approximately 50° of the central visual field. Hexagons had a luminance of either 400 cd/m ("on") or 0.0 cd/m^2 ("off"), with the on/off string of each hexagon determined according to a 14-bit M-sequence. The base period for stimulus presentation was 13.3 ms (equivalent to 75 Hz) and the recordings were bandpass filtered (10–100 Hz) to remove extraneous electrical noise.[16] Each recording session lasted 30 seconds, with a minimum of eight sessions required to complete the MF-ERG recording.

From the ERG, the a-wave, b-wave, and flicker peak times and amplitudes were ascertained for each eye and each stimulus condition (Figs. 1d–g) with the exception of the dark-adapted 0.01 ("rod") a-wave, which is not recommended for quantitative analysis.[5] Ratios of the rod-cone and cone b-wave/a-wave amplitudes were calculated. MF-ERG P1 peak times and amplitudes were analyzed using the concentric ring method[16] (Figs. 1h, 1i).

Statistical Analysis

Electrophysiological and OCT results were compared with our preexisting clinical normative databases, comprising data acquired on-site from single eyes of healthy individuals using the same devices used in the study. From these databases, all available healthy subjects of comparable age to the MS cohort were included in the analysis (ERG: $n = 49$ subjects; MF-ERG: $n = 36$ subjects; OCT: $n = 38$ subjects). The age distributions of the MS cohort and the subjects contributing normative data for the ERG, MF-ERG, and OCT analyses are shown in Table 1. Two statistical analyses were performed: first, the functional (electrophysiological) and structural (OCT) results were compared by category (MS versus normal) and optic neuritis (ON) history (positive versus negative) using generalized estimating equation (GEE) models to account for the intereye dependency of within-subject measurements.[17,18] Second, the relationships between ERG and MF-ERG parameters and their presumed structural correlates were quantified in MS patients only, using GEE models otherwise identical to those used in the group comparison. All analyses were adjusted for age. The reported confidence intervals for each coefficient are based on robust standard errors.

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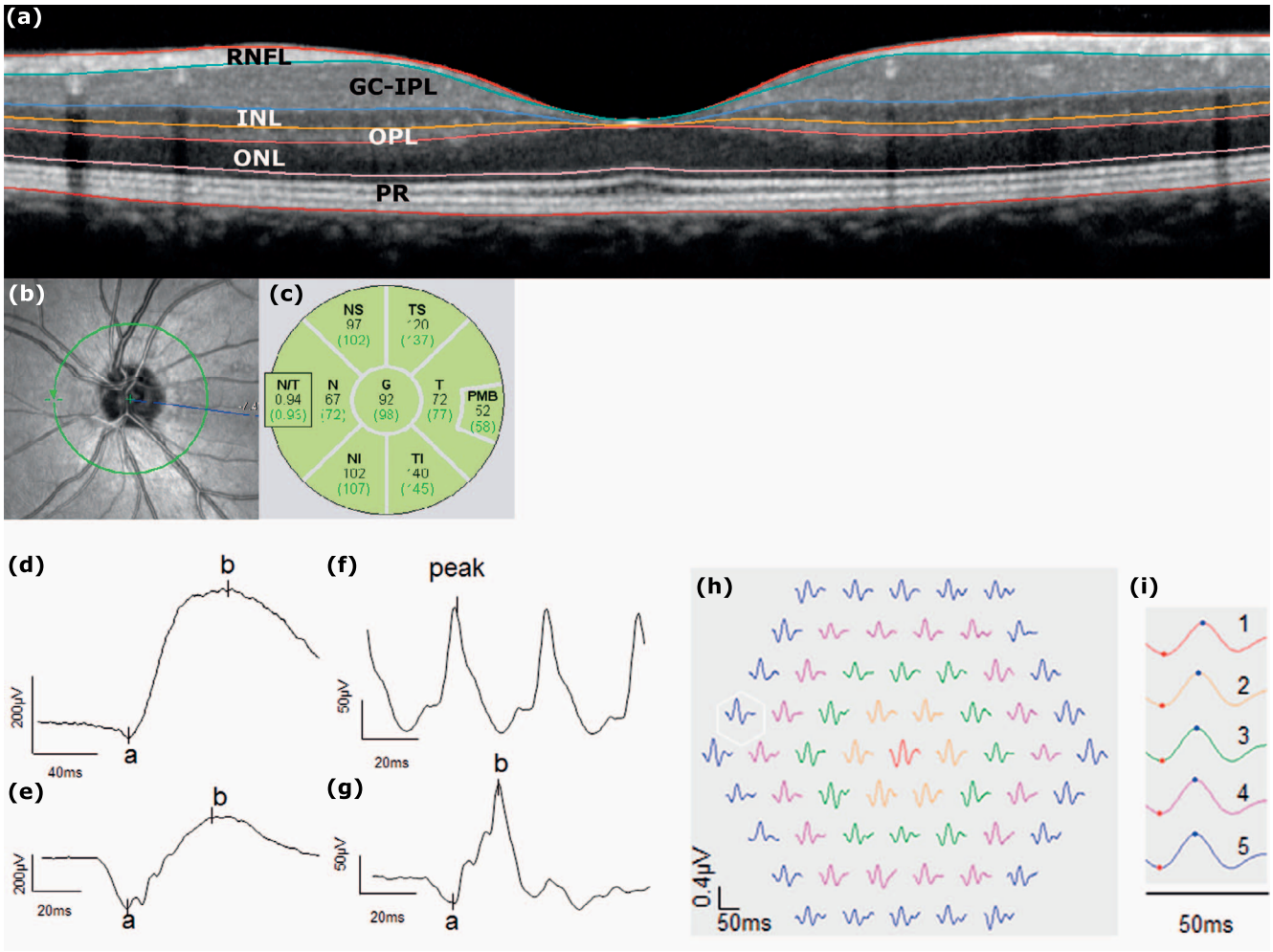

Figure 1. High-resolution macula OCT scan illustrating the retinal layers and complexes included in the analysis (**a**). Circumpapillary OCT scans were centered on the visible center of the optic nerve head (**b**). Analysis of circumpapillary RNFL thickness (left eye). Values for the G, T, and PMB sectors were included in the analysis (**c**). Examples of rod (**d**), rod-cone (**e**), cone flicker (**f**), and cone (**g**) ERG waveforms, showing the a- and b-waves and flicker peak. The amplitudes and peak times of these variables, with the exception of the rod a-wave parameters, were included in the analysis, along with the ratios of the rod-cone and cone b- and a-wave amplitudes. Example of MF-ERG test using a 61-hexagon stimulus array; the concentric rings used for averaging are color-coded for ease of interpretation (**h**). Averaging the traces results in five normalized waveforms, color-coded to match the traces in (**h**). The normalized P1 amplitude and P1 peak time of each of the resultant waveforms (1–5) were included in the analysis (**i**).

Current knowledge as summarized elsewhere[5] implicates the ERG a-wave as being generated mostly by the photoreceptors (the nuclei of which are found in the ONL) with contributions from the bipolar cells (the nuclei of which are found in the INL), with the b-wave being generated mostly by bipolar cells. The P1 component of the MF-ERG is also generated mostly by the bipolar cells.[7] Therefore, the GEE models quantified the effect of ONL, INL, PR volumes, and ON history on the ERG a-waves, as well as INL and OPL volume and ON history on the ERG b-waves, 30-Hz flicker response peak, and MF-ERG P1 values. OPL was included in the b-wave analyses due to its location as the site of bipolar synaptic terminals; however, IPL was not included in the analyses due to the difficulty in reliably distinguishing it from the adjacent ganglion cell layer (GCL; GCL and IPL are aggregated to GC-IPL in the present work for the same reason).[19–21] In every case, the Benjamini-Hochberg procedure was applied to the P values to correct for multiple testing.[22] P values <0.01 were considered strong evidence of an effect of the pertaining statistical parameter, whereas P values between 0.01 and 0.05 and between 0.05 and 0.10 were considered to represent good and mild evidence,[23] respectively; all other P values were considered nonsignificant. All analyses were performed within R version 3.3.1[24] using the geepack library (version 1.2-1).[25]

Table 1. Age Distributions of the MS Cohort (MS) and the Healthy Individuals Contributing Normative Data for the ERG (Normative ERG), MF-ERG (Normative MF-ERG), and OCT (Normative OCT)

		Mean		Median	
	n	Age, y	SD	Age, y	IQR
MS	32	35.8	10.6	35.0	27.0–44.2
Normative ERG	49	35.4	10.8	31.0	28.0–43.0
Normative MF-ERG	36	36.9	12.5	34.5	27.3–50.8
Normative OCT	38	35.6	12.5	29.5	26.0–46.0

The distributions are described by the mean and SD, and by the median and the IQR. All values are in years.

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Table 2. Results of ERG Examinations in MS Patients (MS) and Healthy Subjects Previously Examined in our Clinic (Normative)

ERG Variable	Category	*n*	Mean	SD	Median	IQR
Rod b-wave AMP, μV	Normative	49	339.518	83.826	342.800	284.900 to 382.300
	MS	63	387.778	70.877	395.400	339.350 to 440.200
Rod b-wave PEAK, ms	Normative	49	77.969	6.937	78.000	74.000 to 80.500
	MS	63	80.365	7.279	80.000	74.500 to 84.750
Rod-cone a-wave AMP, μV	Normative	49	−298.996	65.828	−294.000	−332.700 to −259.900
	MS	63	−329.063	67.921	−328.600	−371.150 to −279.850
Rod-cone a-wave PEAK, ms	Normative	49	14.765	0.511	15.000	14.500 to 15.000
	MS	63	15.286	0.711	15.500	15.000 to 15.500
Rod-cone b-wave AMP, μV	Normative	49	470.598	112.288	483.100	397.500 to 542.900
	MS	63	518.843	86.434	508.400	453.350 to 559.250
Rod-cone b-wave PEAK, ms	Normative	49	52.561	3.813	52.500	50.000 to 55.000
	MS	63	54.548	3.504	54.000	52.000 to 57.000
Cone flicker AMP, μV	Normative	49	136.356	32.964	137.900	114.800 to 151.500
	MS	63	141.040	33.411	134.300	120.100 to 164.850
Cone flicker PEAK, ms	Normative	49	26.929	1.271	26.500	26.000 to 28.000
	MS	63	28.444	1.856	28.500	27.500 to 29.500
Cone a-wave AMP, μV	Normative	49	−47.946	13.096	−47.600	−56.350 to −39.370
	MS	63	−52.435	12.894	−54.610	−61.665 to −41.440
Cone a-wave PEAK, ms	Normative	49	13.929	0.685	14.000	13.500 to 14.500
	MS	63	14.468	0.647	14.500	14.000 to 15.000
Cone b-wave AMP, μV	Normative	49	176.934	40.406	177.700	148.100 to 202.800
	MS	63	182.117	34.745	179.000	157.100 to 204.900
Cone b-wave PEAK, ms	Normative	49	30.051	1.312	30.000	29.000 to 31.000
	MS	63	31.833	1.818	32.000	30.500 to 33.000
Rod-cone b/a-wave ratio	Normative	49	1.584	0.251	1.565	1.418 to 1.695
	MS	63	1.602	0.215	1.557	1.488 to 1.661
Cone b/a-wave ratio	Normative	49	3.787	0.653	3.721	3.242 to 4.288
	MS	63	3.573	0.636	3.427	3.093 to 4.072

For each ERG amplitude (AMP) and peak time (PEAK) parameter, the number of eyes analyzed (*n*) is shown, along with values for mean, SD, median, and IQR. AMP values are given in microvolts, and PEAK in milliseconds.

Results

A total of 32 subjects (22 female) had completed the initial examination of the longitudinal study, the results of which were analyzed on a cross-sectional basis. Of the patient cohort, 19 had relapsing-remitting MS, 11 CIS, and two primary progressive MS (PPMS). Median age was 35 years (range, 21–59 years), whereas median Enhanced Disability Status Scale (EDSS) score was 1.0 (range, 0.0–4.0); median disease duration (as defined by time since first symptoms) was 18.5 months (range, 3–324). Nineteen patients had a clinical history of ON, four of whom had been affected in both eyes sequentially or simultaneously; however, none of the patients had experienced an ON event within the 3 months before examination.[10] Nineteen patients were receiving disease-modifying therapy at the time of the examination. No patients had myelinated retinal nerve fibers visible on OCT or fundus examination. The demographic characteristics of the patient cohort are shown in more detail in Supplementary Table S1 (Supplementary Data). One patient had minimal residual traces of microcystic macular edema (MMO) in one eye (with a history of ON 30 months previously), which were not unambiguously visible on two adjacent OCT slices and therefore did not meet published MMO diagnostic criteria,[26] and so we did not exclude this patient from OCT analysis; no other patients had visible traces of MMO. Another patient had an exotropia; therefore, data pertaining to this eye were excluded from analysis. One patient was unable to perform the MF-ERG and for another patient it was not possible to perform OCT. In total, 63 eyes of 32 patients were analyzed for the ERG, and 61 eyes of 31 patients for both the MF-ERG and OCT. Mean and median values of all ERG, MF-ERG, and OCT parameters are given in Tables 2, 3, and 4, respectively, for both MS and normative cohorts. Mean high- and low-contrast visual acuity (with SD) were –0.088 ± 0.078 logMAR and 0.464 ± 0.194 logMAR, respectively; median values were –0.10 logMAR and 0.33 logMAR, respectively.

The results of the electrophysiology analyses are shown in Tables 5 and 6. Those conditions for which strong, good, or mild evidence of difference between MS patients and normal values are shown in Figures 2 and 3. For the ERG (Table 5; Fig. 2), four of seven measures of peak time showed strong evidence of a difference between MS patients and normative data (rod-cone a-wave, cone flicker, cone a-wave, and cone b-wave), whereas there was mild evidence for a difference in rod-cone b-wave peak time. Inspection of the data confirmed that this difference was due to longer peak times in the MS group (Table 2; Figs. 2c, 2e–h). All of these longer peak times comprised part of the cone or mixed rod/cone response. No differences in rod b-wave peak time were observed. Strong (rod b-wave), good (rod-cone a-wave), and mild (rod-cone b-wave) evidence was observed for amplitude differences between MS patients and normative data; rod-cone a-waves were lower in amplitude (Fig. 2a), and rod b-waves and rod-cone b-waves of higher amplitude (Figs. 2b, 2d), in MS patients. No other differences in ERG amplitudes were observed. We did not find evidence for a difference in rod-cone or cone a-wave/b-wave ratios between MS patients and normative data. MF-ERG results (Table 6; Fig. 3) showed good (rings 3 and 5) and mild (rings 2 and 4) evidence for prolonged P1 peak times in MS patients, without any differences in response amplitudes. Both ERG and MF-ERG results did not differ in eyes with previous ON compared with those without previous ON.

Analysis of the OCT data (Table 7; Fig. 4) showed no significant differences between MS patients and normative data

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Table 3. Results of MF-ERG Examinations in MS Patients (MS) and Healthy Subjects Previously Examined in Our Clinic (Normative)

MF-ERG Variable	Category	*n*	Mean	SD	Median	IQR
Ring 1 AMP, nV/deg^2	Normative	36	45.392	12.452	44.950	37.625–53.250
	MS	61	45.843	10.951	45.900	38.900–51.500
Ring 1 PEAK, ms	Normative	36	33.103	0.957	33.300	32.400–34.100
	MS	61	33.502	1.299	33.300	32.400–34.100
Ring 2 AMP, nV/deg^2	Normative	36	24.308	6.533	22.850	19.800–29.300
	MS	61	24.941	4.843	24.900	21.700–27.700
Ring 2 PEAK, ms	Normative	36	31.956	1.134	31.600	30.800–32.400
	MS	61	32.693	1.167	32.400	32.400–33.300
Ring 3 AMP, nV/deg^2	Normative	36	13.942	3.641	13.350	11.150–15.950
	MS	61	14.495	2.811	14.200	12.400–16.600
Ring 3 PEAK, ms	Normative	36	31.178	1.130	31.200	30.600–31.800
	MS	61	31.946	1.207	31.600	31.600–32.400
Ring 4 AMP, nV/deg^2	Normative	36	9.739	2.362	9.250	8.200–11.200
	MS	61	10.431	2.155	10.200	9.000–11.200
Ring 4 PEAK, ms	Normative	36	31.164	1.042	30.800	30.800–31.600
	MS	61	31.852	1.277	31.600	30.800–32.400
Ring 5 AMP, nV/deg^2	Normative	36	8.425	2.164	7.850	6.700–10.125
	MS	61	8.526	1.999	8.200	7.500–9.300
Ring 5 PEAK, ms	Normative	36	31.139	0.999	30.800	30.600–31.600
	MS	61	31.982	1.358	32.400	30.800–32.400

For each MF-ERG amplitude (AMP) and peak time (PEAK) parameter calculated over five concentric rings (Fig. 1h), the number of eyes analyzed (*n*) is shown, along with values for mean, SD, median, and IQR. AMP values are given in nanovolts per square degree, and PEAK values in milliseconds.

for any of the measured retinal layers/complexes. However, strong evidence of difference between eyes with and without previous ON was found for RNFL-G, -T, and -PMB, as well as GC-IPL; mild evidence of difference was found for the INL. All measures of RNFL and GC-IPL showed reduced thickness or volume after ON (Figs. 4a–d), whereas INL volume was slightly increased (Fig. 4e).

Assessment of the relationships between retinal function and structure in MS patients showed strong (rod-cone a-wave) to good (cone a-wave; rod-cone and cone b-waves) evidence for an association between ERG amplitudes and ONL (a-waves) and INL (b-waves). No significant associations with ERG peak times or with any MF-ERG parameters (data not shown) were observed; all analyses pertaining to PR and OPL were also nonsignificant. We found mild ($P = 0.098$) evidence for an influence of ON history on rod-cone a-wave amplitudes; all other *P* values pertaining to ON history were nonsignificant. The results of this analysis are summarized in Table 8.

Two patients had PPMS (Supplementary Table S1), which is phenotypically distinct from other forms of MS.[14] We therefore repeated all GEE analyses previously described with both PPMS patients excluded, to exclude the remote possibility that their inclusion may have unduly influenced results; the pattern of results was unchanged (data not shown).

Discussion

Our study presents strong evidence for altered outer retinal function in patients with MS. The results did not provide evidence for an influence of ON on the ERG and MF-ERG in our

Table 4. Results of OCT Examinations in MS Patients (MS) and Healthy Subjects Previously Examined in our Clinic (Normative)

OCT Variable	Category	*n*	Mean	SD	Median	IQR
RNFL-G, μm	Normative	38	101.368	6.136	100.500	97.000–105.000
	MS	61	96.180	14.874	98.000	91.000–106.000
RNFL-T, μm	Normative	38	73.000	6.998	74.000	67.250–77.000
	MS	61	63.393	16.128	64.000	54.000–74.000
RNFL-PMB, μm	Normative	38	55.368	5.592	54.000	51.000–59.000
	MS	61	48.066	12.128	49.000	42.000–56.000
GC-IPL, mm^3	Normative	38	0.839	0.055	0.850	0.790–0.878
	MS	61	0.778	0.123	0.800	0.700–0.870
INL, mm^3	Normative	38	0.351	0.035	0.350	0.340–0.370
	MS	61	0.352	0.032	0.350	0.330–0.370
OPL, mm^3	Normative	38	0.292	0.038	0.290	0.270–0.310
	MS	61	0.298	0.042	0.290	0.270–0.310
ONL, mm^3	Normative	38	0.698	0.070	0.690	0.670–0.718
	MS	61	0.671	0.078	0.680	0.620–0.740
PR, mm^3	Normative	38	0.774	0.019	0.770	0.760–0.790
	MS	61	0.766	0.021	0.770	0.750–0.780

For each structural parameter, the number of eyes analyzed (*n*) is shown, along with values for mean, SD, median, and IQR. RNFL-G, RNFL-T, and RNFL-PMB values are given in micrometers, whereas volumes of the GC-IPL, INL, OPL, ONL, and PR over the 1-, 2.22-, and 3.45-mm ETDRS grid are given in cubic millimeters.

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Table 5. Results of GEE Models for ERG Amplitude (AMP) and peak time (PEAK) Variables According to MS Status (MS Versus Healthy) and ON History (Positive Versus Negative), Adjusted for Age and Including Coefficients, 95%-Wald Confidence Intervals (CI) of the Coefficients, and Corrected *P* Values (Benjamini-Hochberg)

ERG Variable	Covariate	Coefficient	95% CI	*P*, Corrected
Rod b-wave AMP	MS status	52.145	19.856 to 84.434	**0.008**
	ON history	−9.936	−41.626 to 21.754	0.746
Rod b-wave PEAK	MS status	2.517	−0.815 to 5.848	0.234
	ON history	−0.340	−4.023 to 3.344	0.921
Rod-cone a-wave AMP	MS status	−37.731	−67.559 to −7.903	**0.040**
	ON history	22.323	−6.511 to 51.158	0.530
Rod-cone a-wave PEAK	MS status	0.530	0.258 to 0.802	**0.001**
	ON history	−0.061	−0.261 to 0.140	0.746
Rod-cone b-wave AMP	MS status	53.023	8.065 to 97.981	**0.056**
	ON history	−13.469	−61.365 to 34.427	0.748
Rod-cone b-wave PEAK	MS status	1.793	0.220 to 3.365	**0.057**
	ON history	0.394	−0.379 to 1.167	0.643
Cone flicker AMP	MS status	6.773	−7.303 to 20.850	0.518
	ON history	−4.446	−12.312 to 3.420	0.643
Cone Flicker PEAK	MS status	1.429	0.743 to 2.115	**<0.001**
	ON history	0.198	−0.212 to 0.607	0.643
Cone a-wave AMP	MS status	−4.672	−10.245 to 0.900	0.193
	ON history	0.401	−5.121 to 5.922	0.921
Cone a-wave PEAK	MS status	0.586	0.279 to 0.894	**0.001**
	ON history	−0.147	−0.361 to 0.066	0.530
Cone b-wave AMP	MS status	6.144	−9.434 to 21.722	0.593
	ON history	−1.760	−14.416 to 10.895	0.910
Cone b-wave PEAK	MS status	1.629	0.861 to 2.396	**<0.001**
	ON history	0.354	−0.152 to 0.859	0.530
Rod-cone b-/a-wave ratio	MS status	0.007	−0.093 to 0.106	0.896
	ON history	0.033	−0.008 to 0.073	0.530
Cone b-/a-wave ratio	MS status	−0.226	−0.515 to 0.064	0.228
	ON history	0.038	−0.272 to 0.349	0.910

Corrected *P* values that represent strong, good, or mild evidence of difference between MS patients and normative data are highlighted with bold text. A total of 63 eyes from 32 MS patients were analyzed, along with 49 eyes from 49 healthy individuals.

Table 6. Results of GEE models for MF-ERG amplitude (AMP) and peak time (PEAK) Variables Over Five Concentric Rings According to MS Status (MS Versus Healthy) and ON History (Positive Versus Negative), Adjusted for Age and Including Coefficients, 95%-Wald CIs of the Coefficients, and Corrected *P* Values (Benjamini-Hochberg)

MF-ERG Variable	Covariate	Coefficient	95% CI	*P*, Corrected
Ring 1 AMP	MS status	1.138	−4.315 to 6.590	0.803
	ON history	−2.541	−7.953 to 2.870	0.643
Ring 1 PEAK	MS status	0.255	−0.312 to 0.823	0.536
	ON history	0.370	−0.381 to 1.122	0.643
Ring 2 AMP	Category	0.496	−2.192 to 3.184	0.803
	ON history	0.194	−1.294 to 1.682	0.910
Ring 2 PEAK	MS status	0.609	0.023 to 1.195	**0.087**
	ON history	0.359	−0.250 to 0.969	0.643
Ring 3 AMP	MS status	0.244	−1.217 to 1.704	0.803
	ON history	0.794	−0.355 to 1.944	0.530
Ring 3 PEAK	MS status	0.686	0.143 to 1.229	**0.040**
	ON history	0.224	−0.089 to 0.537	0.530
Ring 4 AMP	MS status	0.552	−0.484 to 1.589	0.471
	ON history	0.349	−0.703 to 1.402	0.746
Ring 4 PEAK	MS status	0.624	0.083 to 1.165	**0.057**
	ON history	0.144	−0.261 to 0.548	0.746
Ring 5 AMP	MS status	0.109	−0.839 to 1.056	0.854
	ON history	0.015	−0.765 to 0.795	0.970
Ring 5 PEAK	MS status	0.758	0.218 to 1.298	**0.023**
	ON history	0.136	−0.300 to 0.572	0.746

Corrected *P* values that represent strong, good, or mild evidence of difference between MS patients and normative data are highlighted with bold text. A total of 61 eyes from 31 MS patients were analyzed, along with 36 eyes from 36 healthy individuals.

patient cohort, which is consistent with outer retinal dysfunction as a primary manifestation of MS. Although this interpretation of our data concords with previous ERG (but not MF-ERG) findings in MS patients without previous ON,[11] we are nevertheless unable to exclude the possibility that the relatively modest sample size of the MS ON subgroups in our study may have contributed to this negative result. Previous work has proposed primary retinal pathology in a subset of MS patients with atypical OCT findings of the outer retina (representing approximately 10% of the patient population at that center)[10]; our results extend this finding and suggest that effects of MS on the outer retina may be a more widespread phenomenon than previously thought. Despite our OCT device being capable of acquiring scans with an optical axial resolution of 7 μm,[27] we did not record any changes to the corresponding retinal layers due to MS.

Previous work examining the ERG in MS patients has produced apparently contradictory findings, with some authors reporting normal ERG results[28–30] and others a range of abnormalities.[11–13,31–34] Significantly, those studies that reported normal ERG findings in MS patients analyzed only response amplitudes, with no peak time values presented.[28–30] Previous studies showing changes to the peak time of the cone[12,34] or mixed rod/cone[11,34] ERG responses are unanimous in recording delayed responses relative to normative values, as described here.

With regard to response amplitudes, previous results are mixed, with studies showing normal,[28–30] reduced,[31,32] or increased[13] ERG amplitudes in MS patients relative to control subjects; none of these studies also analyzed peak time. In this context, our results showing mostly normal amplitudes (with increased or decreased amplitudes for some conditions) are not contrary to previous studies. We are unaware of any previous analysis of b-wave/a-wave amplitude ratios in MS patients; in the present work, these ratios did not differ significantly between MS patients and normative data. It seems that ERG peak times are more likely than response amplitudes or b-wave/a-wave amplitude ratios to be abnormal in MS patients and may thus represent a more sensitive indicator of outer retinal dysfunction.

To our knowledge, the present study is the first to show MF-ERG abnormalities in a typical MS cohort. In MS patients without previous ON, MF-ERG has been recorded as normal,[11] whereas five of seven patients with atypical OCT findings (reduced macular thickness in the presence of normal RNFL and GC-IPL thickness) were found to exhibit reduced P1 amplitudes.[10] In contrast, our patient cohort (which was mixed with regard to MS type and history of ON) was found to have mostly prolonged P1 peak times without any significant differences in P1 response amplitudes. No differences between eyes with and without a history of ON were observed. Abnormal P1 peak times suggest dysfunction of the on-bipolar cells[7] and are hypothesized to reflect damage between cone inner segments and postsynaptic membranes of bipolar cells,[35] although note that dysfunction confined to the photoreceptors may also affect P1.[35]

These effects of MS are apparent at the level of the photoreceptors and bipolar cells of the outer retina, the first- and second-order retinal neurons, respectively. It is not possible from our data to infer the physiological mechanisms

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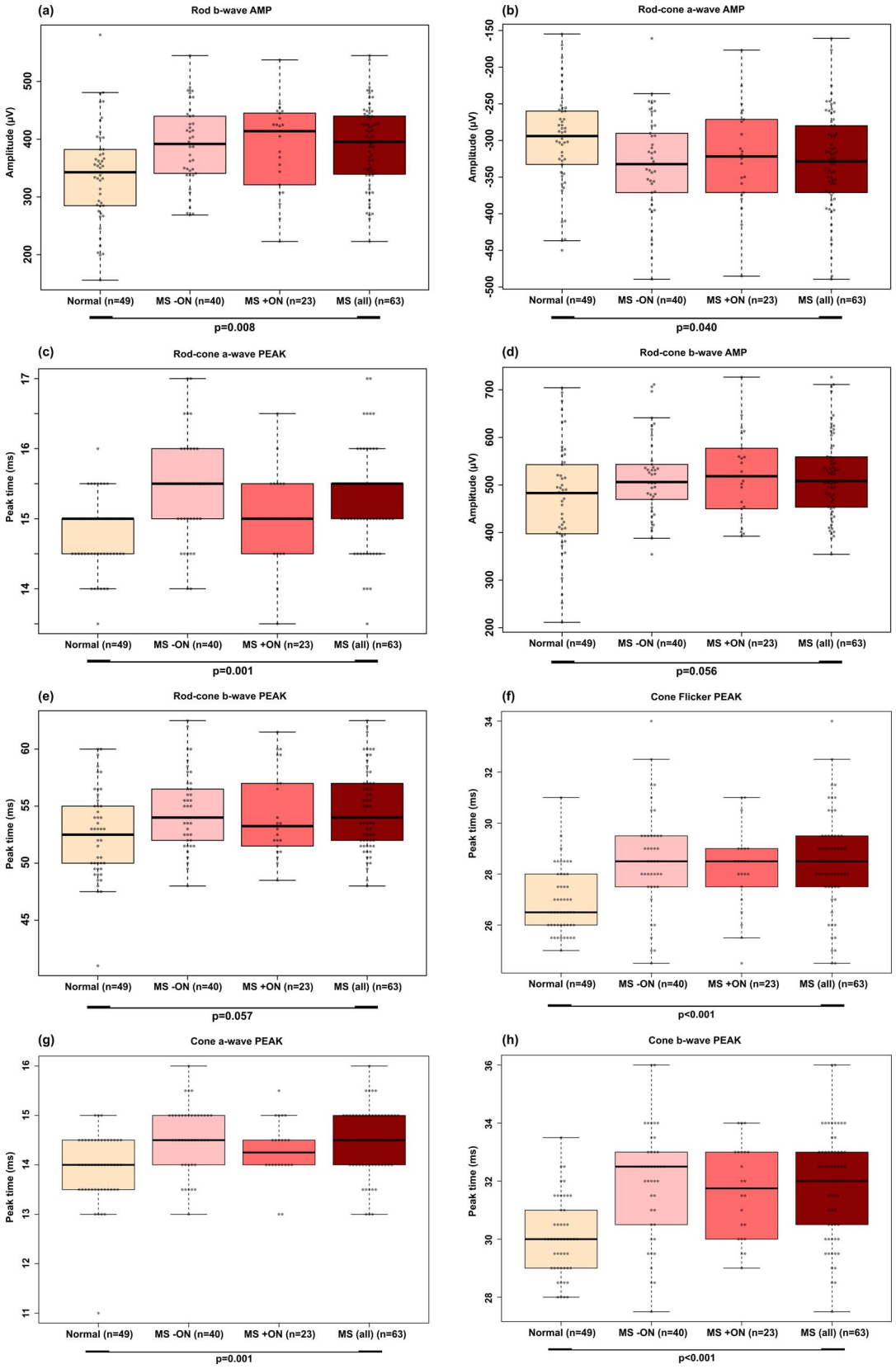

Figure 2. (**a–h**) *Boxplots* showing ERG peak time (PEAK) and amplitude (AMP) results in MS patients. For brevity, only those parameters in which strong, good, or mild evidence of a difference between MS patients and normal values are displayed; the data for all parameters are shown in Table 2. *Leftmost bars* show preexisting clinical normative values (Normal), followed by results from MS eyes without a history of ON (MS −ON), MS patients with a history of ON (MS +ON), and all MS eyes (MS [all]). For each *bar*, the number of eyes analyzed (*n*) is displayed. On each *plot*, the corrected *P* value resulting from the comparison of the MS cohort with normative data is displayed. Median values and interquartile ranges (IQRs) are indicated by *horizontal lines* and *boxes*, respectively; *whiskers* show the lowest and highest data points still within 1.5 IQR of the lower and upper quartiles. Individual data points are represented by *gray dots*. Peak times are displayed in milliseconds, amplitudes in microvolts.

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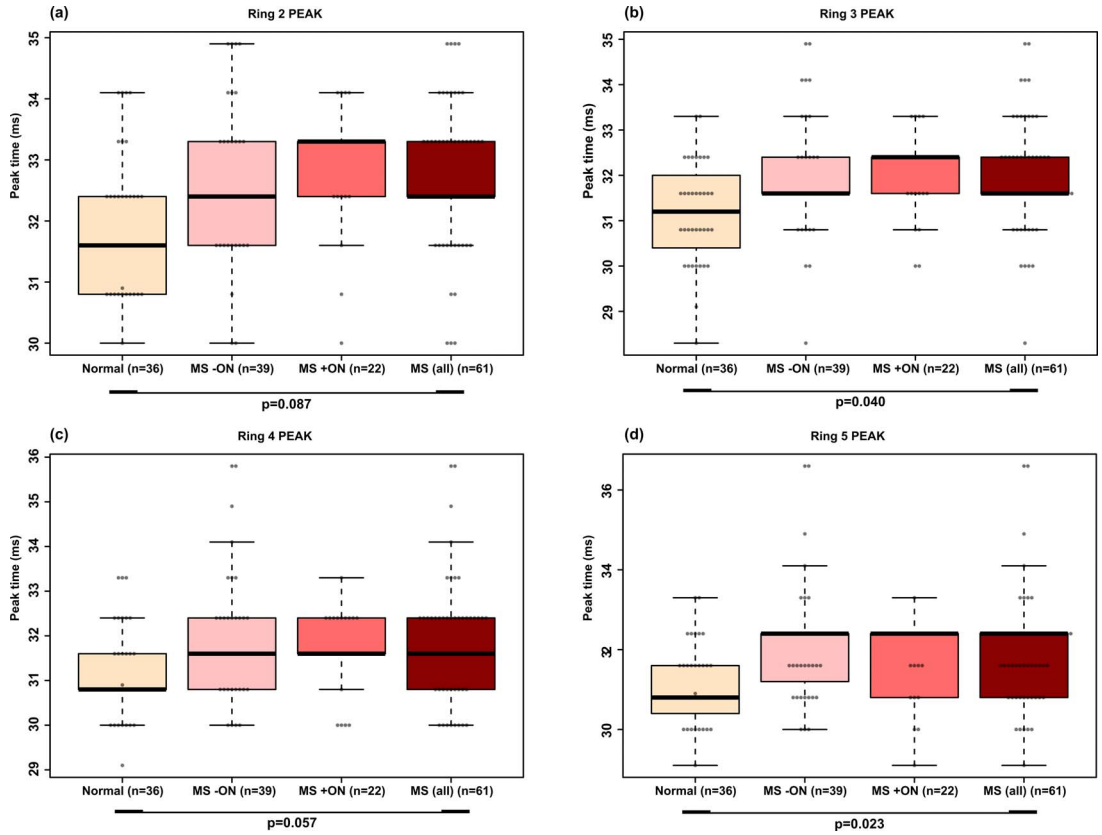

Figure 3. (**a–d**) *Boxplots* showing MF-ERG P1 peak time (PEAK) results in MS patients. For brevity, only those parameters in which strong, good, or mild evidence of a difference between MS patients and normative data are displayed; the data for all parameters are shown in Table 3. *Leftmost bars* show preexisting clinical normative values (Normal), followed by results from MS eyes without a history of ON (MS −ON), MS patients with a history of ON (MS +ON), and all MS eyes (MS [all]). For each *bar*, the number of eyes analyzed (n) is displayed. On each *plot*, the corrected *P* value resulting from the comparison of the MS cohort with normative data is displayed. Median values and IQRs are indicated by *horizontal lines* and *boxes*, respectively; *whiskers* show the lowest and highest data points still within 1.5 IQR of the lower and upper quartiles. Individual data points are represented by *gray dots*. P1 peak times are displayed in milliseconds. Note that all P values pertaining to MF-ERG amplitude (AMP) variables were nonsignificant, and thus no MF-ERG AMP plots are displayed.

Table 7. Results of GEE Models for OCT Variables According to MS Status (MS Versus Healthy) and ON History (Positive Versus Negative), Adjusted for Age and Including Coefficients, 95%-Wald CIs of the Coefficients, and Corrected *P* Values (Benjamini-Hochberg)

OCT Variable	Covariate	Coefficient	95% CI	*P*, Corrected
RNFL-G	MS status	0.697	−3.302 to 4.697	0.977
	ON history	−16.492	−25.151 to −7.833	**<0.001**
RNFL-T	MS status	−2.911	−8.341 to 2.520	0.683
	ON history	−18.402	−27.248 to −9.556	**<0.001**
RNFL-PMB	MS status	−1.917	−5.867 to 2.033	0.683
	ON history	−14.667	−20.799 to −8.534	**<0.001**
GC-IPL	MS status	−0.002	−0.034 to 0.030	0.984
	ON history	−0.164	−0.226 to −0.101	**<0.001**
INL	MS status	−0.004	−0.019 to 0.011	0.946
	ON history	0.012	0.001 to 0.023	**0.053**
OPL	MS status	0.000	−0.016 to 0.016	0.984
	ON history	0.017	−0.003 to 0.037	0.117
ONL	MS status	−0.027	−0.062 to 0.008	0.528
	ON history	0.002	−0.019 to 0.022	0.884
PR	MS status	−0.011	−0.021 to −0.001	0.190
	ON history	0.007	−0.001 to 0.015	0.117

Corrected *P* values that represent strong, good, or mild evidence of difference between MS patients and normative data are highlighted with bold text. A total of 61 eyes from 31 MS patients were analyzed, along with 38 eyes from 38 healthy individuals.

underlying these abnormalities; however, the lack of any observed differences in eyes with and without previous ON (with the caveat that the power of our analysis may be reduced due to the relatively small size of the MS ON subgroups) suggests that retrograde transsynaptic degeneration consequent to inflammation and demyelination of the optic nerve as a source of the functional deficits described here is unlikely. This is reinforced by previous studies showing abnormal ERG findings in MS patients *without* previous ON,[11] and suggesting that retrograde transsynaptic degeneration in MS does not affect the INL or more distal layers.[36] The absence of myelin in human retinae, confirmed in our entire cohort by ophthalmologic examination, also suggests that an autoimmune response to myelin antigens is unlikely as a potential mediator of the changes described here. Given that glutamate is the most prevalent neurotransmitter throughout the retina and visual pathway,[37] and that the glutamergic pathway is frequently dysfunctional in MS,[38] glutamergic abnormalities could be considered as a possible explanation for outer retinal dysfunction in our cohort; further studies would be needed to confirm or exclude this possibility.

Despite the results showing evidence of retinal dysfunction in MS patients, OCT analysis showed no significant differences between patients and normative values for any of the analyzed retinal layers or complexes. This may be due to reduced statistical power consequent to the relatively low number of patients examined in the present study, as previous work with

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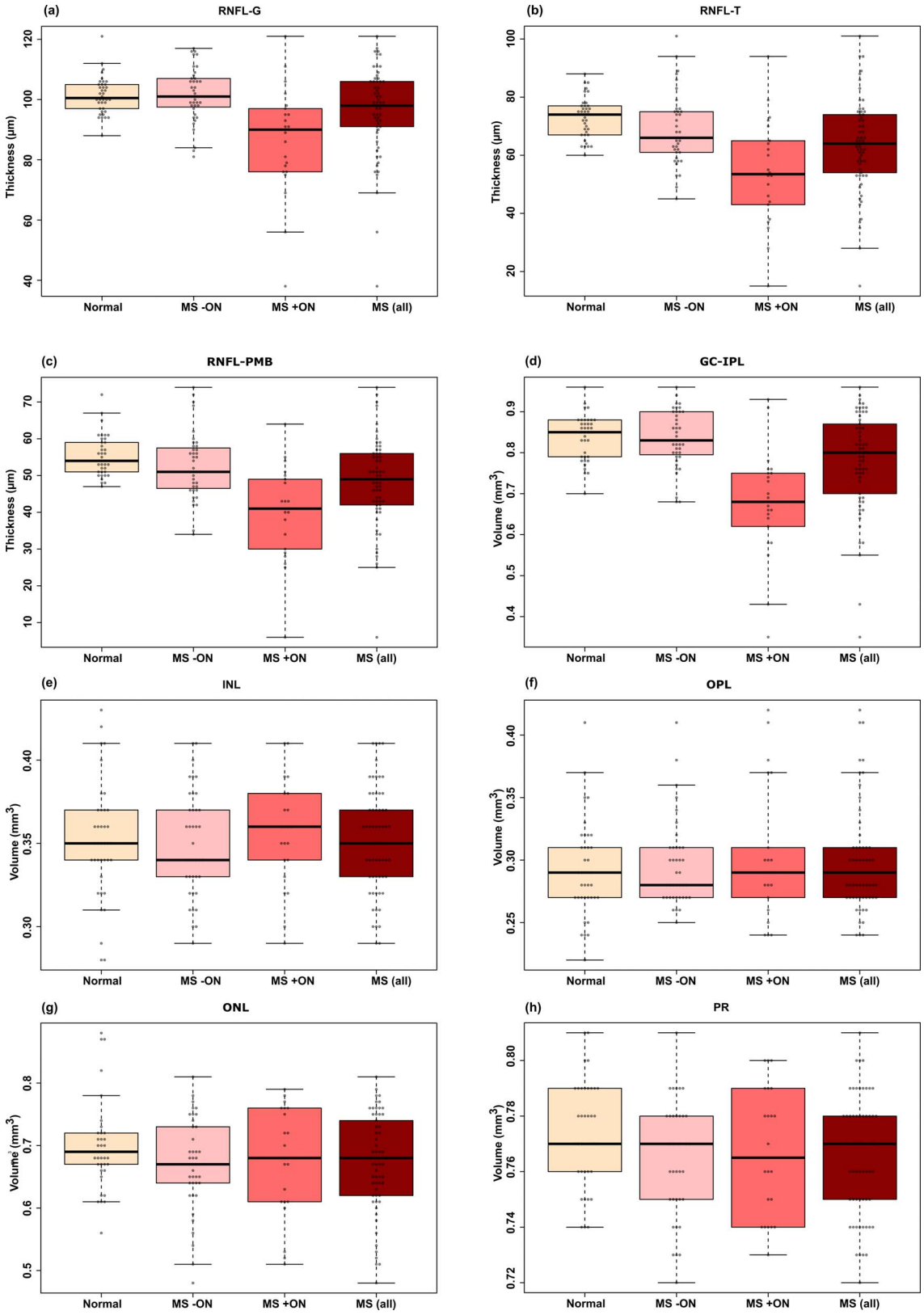

Figure 4. (**a–h**) *Boxplots* showing OCT results in MS patients. *Leftmost bars* show preexisting clinical normative values (Normal), followed by results from MS eyes without a history of ON (MS −ON), MS patients with a history of ON (MS +ON), and all MS eyes (MS [all]). For each *bar*, the number of eyes analyzed (*n*) is displayed. On each *plot*, the corrected *P* value resulting from the comparison of the MS cohort with normative data is displayed. Median values and IQRs are indicated by *horizontal lines* and *boxes*, respectively; *whiskers* show the lowest and highest data points still within 1.5 IQR of the lower and upper quartiles. Individual data points are represented by *gray dots*. Circumpapillary RNFL-G, RNFL-T, and RNFL-PMB thickness values are given in microns, whereas volumes for the remaining retinal layers and complexes over the 1-, 2.22-, and 3.45-mm ETDRS grid are given in cubic millimeters.

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Table 8. Results of GEE Models Quantifying the Influence of Retinal Structure and Previous ON on ERG Amplitudes (AMP) and Peak Times (PEAK) in MS Patients, Adjusted for Age and Including Coefficients, 95%-Wald CIs of the Coefficients, and Corrected *P* Values (Benjamini-Hochberg)

ERG Variable	Covariate	Coefficient	95% CI	*P*, Corrected
Rod-cone a-wave AMP	INL	−392.057	−745.048 to −39.065	0.118
	ONL	−370.087	−572.005 to −168.168	**0.001**
	PR	−175.531	−950.464 to 599.401	0.876
	ON history	27.259	3.523 to 50.996	**0.098**
Rod-cone a-wave PEAK	INL	0.754	−2.891 to 4.399	0.685
	ONL	0.556	−1.064 to 2.176	0.610
	PR	−2.778	−8.386 to 2.831	0.876
	ON history	−0.131	−0.278 to 0.015	0.159
Cone a-wave AMP	INL	−20.571	−103.985 to 62.843	0.685
	ONL	−55.745	−97.764 to −13.727	**0.019**
	PR	5.264	−174.944 to 185.472	0.954
	ON history	0.610	−4.727 to 5.947	0.823
Cone a-wave PEAK	INL	1.549	−3.495 to 6.593	0.685
	ONL	0.746	−2.120 to 3.612	0.610
	PR	−1.810	−7.940 to 4.319	0.876
	ON history	−0.142	−0.341 to 0.058	0.218
Rod b-wave AMP	INL	192.965	−420.627 to 806.557	0.663
	OPL	−2.947	−459.671 to 453.778	0.990
	ON history	−12.964	−44.388 to 18.461	0.620
Rod b-wave PEAK	INL	−15.870	−68.226 to 36.487	0.663
	OPL	−6.621	−50.023 to 36.781	0.918
	ON history	0.093	−3.801 to 3.986	0.963
Rod-cone b-wave AMP	INL	649.359	120.175 to 1178.544	**0.049**
	OPL	−410.882	−1011.008 to 189.244	0.359
	ON history	−19.616	−64.295 to 25.062	0.620
Rod-cone b-wave PEAK	INL	6.927	−5.942 to 19.795	0.583
	OPL	−9.915	−21.977 to 2.146	0.359
	ON history	0.580	−0.152 to 1.312	0.392
Cone b-wave AMP	INL	221.501	74.825 to 368.176	**0.018**
	OPL	−69.846	−283.202 to 143.510	0.782
	ON history	−3.636	−14.626 to 7.354	0.620
Cone b-wave PEAK	INL	1.467	−5.865 to 8.798	0.695
	OPL	−2.226	−5.031 to 0.580	0.359
	ON history	0.213	−0.063 to 0.489	0.392

The effect of ONL, PR, and INL on ERG a-waves and of INL and OPL on ERG b-waves and flicker responses is analyzed; this reflects current knowledge of the likely cellular origins of ERG components (see main text for details). Corrected *P* values that represent strong, good, or mild evidence of difference between MS patients and normative data are highlighted with bold text. A total of 61 eyes from 31 MS patients were analyzed for the models quantifying the influence of retinal structure and previous ON on the ERG. All *P* values pertaining to the influence of retinal structure and previous ON on the MF-ERG were nonsignificant (after analyzing 59 eyes from 30 MS patients) and are omitted for brevity (see main text for details).

considerably larger cohorts has documented inner retinal thinning in MS patients.[10,36] Despite this caveat, our findings regarding the outer retina are essentially confirmed by a recent meta-analysis comparing retinal layer thicknesses in MS eyes with and without previous ON with healthy control subjects.[39] After pooling data from multiple studies and many eyes (with individual MS subgroups ranging in size from 321 to 695 eyes), this analysis found that combined OPL-ONL thickness appears indistinguishable from normal in MS eyes both with and without previous ON, whereas MS eyes with previous ON had OPL-ONL thickness approximately 1 μm thicker than MS eyes without previous ON.[39] INL thickness also appears normal in MS eyes without previous ON,[39] as in the present study. When comparing eyes with previous ON to those without, we found strong evidence for reduction of RNFL thickness (RNFL-G, RNFL-T, and RNFL-PMB) and GCL-IPL volume, as expected based on previous reports,[39–41] as well as mild evidence for increased INL volume, as described elsewhere.[39]

Of particular interest was that no volume differences of the INL and outer retinal layers were observed between MS patients and normative data, despite these layers being the presumed origin of many of the abnormal ERG responses that were documented; in other words, we observed no structural changes corresponding to the measured functional abnormalities. There are at least two possible reasons for this apparent discrepancy. First, recent research into the INL has suggested that its volume in MS patients is dynamic; untreated patients had significantly thicker INL than healthy subjects, which remained constant in those patients who commenced ineffective therapy but normalized in those patients in whom therapy was effective (as evidenced by no clinical relapses or new MRI lesions during the follow-up period).[9] Alternatively, other authors have proposed that MS patients may exhibit *thinning* of the INL.[10,42] In our study, the wide range of INL volumes in both the normative data and patient cohort may have contributed to the lack of detectable differences between the two groups (Fig. 3e). Additionally, the electroretinographic abnormalities we recorded may reflect simply dysfunction of the corresponding cells, rather than atrophy, which would be more likely to be evidenced by reduced volume/thickness on OCT (e.g., as in the case of atrophy and thinning of retinal ganglion cells after ON[19]). Dysfunction also may be more compatible with the relatively subtle nature of the ERG changes documented here. An additional consideration is that

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the ERG reflects the response of the entire retina, whereas the OCT outcome measures are derived from an area of central retina approximately 12° in diameter, and so we cannot exclude the possibility that the retinal dysfunction described here disproportionately affects the peripheral retina.

Analysis of the relationships between ERG parameters and their presumed cellular origins in MS patients showed good to strong evidence of an association between rod/cone and cone ERG a- and b-wave amplitudes and the ONL and INL, respectively. Given that our data show that ERG peak times are more likely than amplitudes to be abnormal in MS, the lack of correlation between OCT findings and ERG peak times could be considered surprising. However, this is consistent with dysfunctional, but not atrophic, bipolar cells and photoreceptors. No significant associations were found between MF-ERG and OCT findings despite the MF-ERG stimulus being closer in size to the OCT ETDRS grid (although still approximately four times larger) than the pan-retinal ERG stimulus. Given our results, it is likely that the ERG (rather than MF-ERG) is the more sensitive method for detecting outer retinal dysfunction in MS patients.

Based on the results of the cross-sectional analysis presented here and recent work suggesting that ERG amplitudes (but not peak times) are influenced by electrode placement,[43] we propose to focus our eventual longitudinal data analysis on the electrophysiological time-to-peak values rather than response amplitudes. Reducing the number of variables analyzed will also serve to increase the statistical power of future analyses, an important consideration given the large number of variables generated by an electrophysiological test battery.

Our study has clear limitations, namely the lack of a formally enrolled cohort of control subjects and the relatively low number of MS patients examined ($n = 32$). The duration of the study visits (up to 4.5 hours) and the longitudinal nature of the study made the recruitment of a specific cohort of healthy subjects unfeasible. The clinical normative values used had the advantage of being acquired before commencing the present study, preventing any possibility of selection bias. Mitigating against our modestly sized patient cohort, our use of GEE for statistical analysis enabled us to include both eyes of most MS patients in the analysis (due to the model accounting for intereye correlations of within-subject measurements[17]), an approach that ensured the actual number of eyes analyzed exceeded those examined in other studies[12] and avoided potential statistical issues caused by analyzing both eyes of MS patients without controlling for such intereye correlations.[11,34]

In summary, we have shown changes to outer retinal function in patients with MS and CIS, without detectable structural abnormality of the relevant retinal layers. Our data show that structurally normal retinae cannot be assumed to be functionally normal in MS patients. The changes are relatively subtle; however, most of our patients also had early or benign disease (as evidenced by the median EDSS score of 1.0). Further cross-sectional studies with a larger cohort including more severely affected patients will be necessary to confirm whether the degree of retinal dysfunction is related to the severity of disease activity. Analysis of electrophysiological peak times, rather than response amplitudes, may be the most promising approach for future work.

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